Journal of Pharmaceutical and Biomedical Analysis 114 (2015) 305–311

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Myoglobin microplate assay to evaluate prevention of protein peroxidation

Sara S. Marques, Luís M. Magalhães*, Ana I.P. Mota, Tânia R.P. Soares[1], Barbara Korsak[2], Salette Reis, Marcela A. Segundo

UCIBIO, REQUIMTE, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, Rua de Jorge Viterbo Ferreira, 228, Porto 4050-313, Portugal

ARTICLE INFO

Article history:
Received 19 March 2015
Received in revised form 2 June 2015
Accepted 5 June 2015
Available online 10 June 2015

Keywords:
Myoglobin
Peroxyl radical
Protein oxidation
Microplate
Statins
β-blockers

ABSTRACT

The current therapeutic strategies are based on the design of multifunctional drug candidates able to interact with various disease related targets. Drugs that have the ability to scavenge reactive oxygen species (ROS), beyond their main therapeutic action, may prevent the oxidative damage of biomolecules. Therefore, analytical approaches that monitor in a continuous mode the ability of drugs to counteract peroxidation of physiologically relevant biotargets are required.

In the present work, a microplate spectrophotometric assay is proposed to evaluate the ability of selected cardiovascular drugs, including angiotensin-converting enzyme (ACE) inhibitors, β -blockers and statins to prevent protein peroxidation. Myoglobin, which is a heme protein, and peroxyl radicals generated from thermolysis of 2,2′-azo-bis(2-amidinopropane) dihydrochloride at 37 °C, pH 7.4 were selected as protein model and oxidative species, respectively. Myoglobin peroxidation was continuously monitored by the absorbance decrease at 409 nm and the ability of drugs to counteract protein oxidation was determined by the calculation of the area under the curve upon the myoglobin oxidation. Fluvastatin (AUC_{50} = 12.5 ± 1.2 μM) and enalapril (AUC_{50} = 15.2 ± 1.8 μM) showed high ability to prevent myoglobin peroxidation, providing even better efficiency than endogenous antioxidants such as reduced glutathione. Moreover, labetalol, enalapril and fluvastatin prevent the autoxidation of myoglobin, while glutathione showed a pro-oxidant effect.

1. Introduction

The multi-target drug design concept is based on the integration of multiple pharmacophores into a single drug molecule in order to make it active within several biological mechanisms, with the advantages of reduced molecularity, absence of drug–drug interactions and improved pharmacokinetics and pharmacodynamics. This research field has been applied to neurodegenerative and cardiovascular diseases where multi-target strategies are a promising alternative to the classical "one target-one drug" approach [1]. Drugs that have the ability to scavenge reactive oxygen species (ROS), which were implicated in the development of several oxidative-stress related human diseases, have demonstrated beneficial clinical effects, usually named as pleiotropic effects [2]. For this reason, the assessment of antioxidant capacity of drugs and drug candidates is a relevant analytical step to establish their therapeutic profile [1].

Several methodologies for assessment of antioxidant properties have been applied to pharmaceutical compounds, including direct detection of free radical scavenging by electron spin resonance, reduction of synthetic radicals and of metal species, as well as the determination of biomarkers of oxidative stress [3]. Among these methods, the oxygen radical absorbance capacity (ORAC) has become a popular antioxidant assay because peroxyl radicals are employed as reactive species and they represent the main lipid peroxidation propagators [4]. ORAC assay was originally proposed using 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH) as a thermal radical initiator to provide a steady flux of peroxyl radicals to oxidize the model protein β-phycoerythrin which is a fluorescent probe [5]. Drugs with antioxidant properties compete with the probe for the radicals, preventing or retarding probe oxidation.

* Corresponding author. Fax: +351 226093483.
E-mail addresses: luismagalhaes@ff.up.pt, luismamagalhaes@gmail.com (L.M. Magalhães).
[1] REQUIMTE, Lab. of Pharmacology, Department of Drug Sciences, Faculty of Pharmacy, University of Porto, Porto, Portugal.
[2] Department of Chemistry, University of Aveiro, Aveiro, Portugal.

http://dx.doi.org/10.1016/j.jpba.2015.06.006
0731-7085/

However, β-phycoerythrin demonstrated several disadvantages, including the interactions of antioxidants with this protein, its reduced photostability, variations between lot preparations and relatively high cost [4]. During the last decade, other oxidation-sensitive probes with fluorescent (fluorescein [6], *p*-aminobenzoic acid [7], long-wavelength fluorophor nile blue [8]) or absorbance properties (pyrogallol red [9], alizarin red [10]) have been proposed for reaction monitoring. Nevertheless, as the ORAC values are strongly dependent of the selected probe [10], the physiological meaning of ORAC values are limited because these probes do not represent any biological target susceptible to oxidative damage. In fact, a useful technique for the reliable evaluation of antioxidant properties should work at conditions that are as physiologically relevant and as close as possible to the biological system where they will exert the antioxidant effect.

Low density lipoproteins (LDL) have been used as biotarget of peroxidation assays. However, the inter-individual variability and the complexity of LDLs production ruled out this methodology for routine/screening assays [4]. Antioxidant assays based on the inhibition of protein oxidation mediated by peroxyl radicals have been developed using human and bovine serum albumin [11], catalase [12], alkaline phosphatase [13] and citrate synthase [14]. Human serum albumin has also been used as biological target for other reactive oxygen species as hypochlorite [15]. The protein oxidation is determined after a fixed reaction time by the content of carbonyl and hydroperoxide groups formation [11,12], protein size by the band pattern of SDS-PAGE [13], enzymatic activity [12,13], dityrosine [14] and protein chloramines [15] formation. For continuous monitoring of protein oxidation, loss of intrinsic fluorescence of the aromatic residues of serum albumin has also been applied, despite the fact that several drugs can interfere in the fluorescence measurements [16].

Terashima et al. [17] proposed the protein myoglobin as a spectrophotometric probe to evaluate antioxidant capacity. Myoglobin denaturation due to peroxidation is reflected in a decrease of absorbance at 409 nm (Soret band) owing to the exposure of the heme group, buried in a hydrophobic pocket within the protein's interior, to the polar aqueous solvent [18]. This probe was further applied toward different reactive species [19] as well as to determine antioxidant properties of flavonoids [20], leafy vegetables and beans [21] and commercially available miso [22]. However, for peroxyl radical scavenging capacity, samples were pre-incubated at 47 °C and the myoglobin protective ratios were calculated from the data after 30 min of reaction, not considering the kinetic information provided by the area under the curve. Myoglobin and hydrogen peroxide have also been used as an oxidation system of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and the antioxidant capacity was assessed by the lag time required to the formation of ABTS radical cation species [23]. This assay was adapted to microplate format for measurement of total antioxidant capacity of human plasma from healthy donors. However, the probe used for antioxidant assessment is ABTS, which does not represent a biological target.

In the present work, a high-throughput microplate assay based on the structural change of myoglobin due to the reaction with peroxyl radicals under physiological conditions (37 °C, pH 7.4) was developed to evaluate the ability of selected cardiovascular drugs to prevent protein peroxidation, including angiotensin-converting enzyme (ACE) inhibitors, β-blockers and statins. The analytical conditions, including the effect of radical generator, drugs and oxidized products in the absorbance measurement were determined. The role of ethanol and DMSO as cosolvents upon the oxidation kinetics of myoglobin and the prevention of autoxidation of myoglobin by cardiovascular drugs was also studied. The ability of drugs to counteract myoglobin peroxidation was compared to the endogenous antioxidants such as reduced glutathione and taurine.

2. Materials and methods

2.1. Reagents

All chemicals used were of analytical reagent grade with no further purification. 2,2′-Azobis-(2-methylpropionamidine) dihydrochloride (AAPH), atenolol, enalapril maleate salt, L-glutathione reduced, labetalol hydrochloride, myoglobin from equine skeletal muscle (ref. M0630), potassium phosphate monobasic, pravastatin sodium salt hydrate, (±)-propranolol hydrochloride and taurine were obtained from Sigma–Aldrich (St. Louis, MO). Fluvastatin sodium salt, lovastatin and simvastatin were obtained from Cayman Chemical Company (Michigan, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was obtained from Fluka (Buchs, Switzerland). Water from arium water purification system (resistivity > 18 MΩ cm, Sartorius, Goettingen, Germany) was used for preparation of all solutions. Ethanol absolute pro analysis from Panreac (Barcelona, Spain) and DMSO from Sigma–Aldrich (St. Louis, MO) were used as cosolvents.

2.2. Myoglobin stock solution

Myoglobin stock solution (c.a. 500 μg/mL) was prepared by dissolving the protein in phosphate buffer solution (pH 7.4; 50 mM). Before analysis, dilutions of the myoglobin stock solution (between 30 and 300 μg/mL) were prepared in phosphate buffer solution and a linear relationship between the protein concentration and absorbance at 409 nm was established. The calibration procedure was performed every day to assure the integrity of the protein and to provide similar protein concentration for all experiments. Hence, a myoglobin solution (75 μg/mL) that provided an absorbance value of 0.200 ± 0.010 after dilution in the microplate well was prepared (25 μg/mL, concentration in the microplate well). Standard solutions from tested drugs were prepared by dissolving the corresponding solid in phosphate buffer solution.

2.3. Microplate protocol for monitoring myoglobin oxidation

The capacity of drugs to prevent the peroxidation of myoglobin (Mb) was evaluated by high-throughput 96-well microplate using spectrophotometric detection at 409 nm, with wavelength selection by a monochromator (Synergy HT, Bio-Tek Instruments, Winooski, VT, USA). Peroxyl radicals ($ROO^{\bullet}$) were generated by thermo-decomposition of AAPH at 37 °C and at pH 7.4. The microplate layout was tailored to provide results from 6 concentrations of drug, each analyzed in quadruplicate (Supplementary file, Fig. S1). The intrinsic absorption of AAPH at 409 nm, myoglobin (Mb) and of the highest concentration of tested drug was determined in A1–H1, A2–H2 and A3–H3 wells, respectively. The intrinsic absorbance of mixtures (AAPH + drug or myoglobin + drug) was also measured in A4–H4 and A5–H5 wells, respectively, in order to assess the potential interference of drug and/or its oxidation products in the analytical signal of myoglobin during the measurement of its oxidation.

Hence, the microplate myoglobin method was carried out in phosphate buffer (pH 7.4; 50 mM) at 37 °C, where 100 μL of Trolox standard solutions (5.0–100 μM) or drug solution and 100 μL of myoglobin (75 μg/mL) were mixed in each well. After that, microplate was pre-incubated at 37 °C for 30 min. Then, 100 μL of freshly prepared AAPH solution (180 mM) was added and the absorbance at 409 nm was recorded every minute during 240 min, beginning after the first minute of reaction. The control experiment (C_t, Fig. S1), where no protection of myoglobin oxidation is expected, was performed by replacing the drug solution with 100 μL of buffer.